

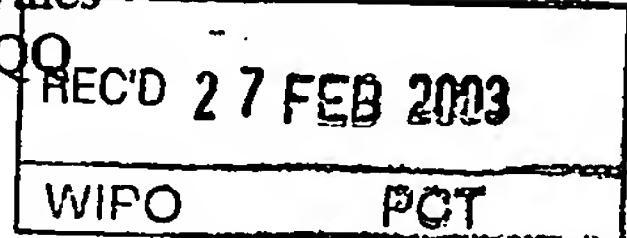
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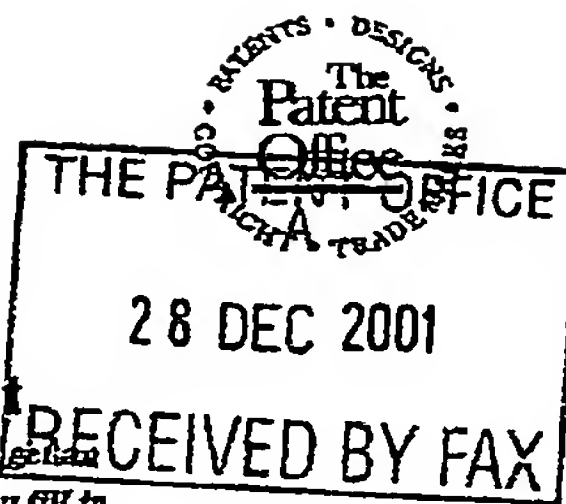
*Stephen Hordley*

Dated 7 February 2003

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311EC01 E684710-1 D02824  
P01/7700 (1.0) 0131026.7

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1. Your reference	P30492/CPA/RMC		
2. Patent application number (The Patent Office will fill in this part)	28 DEC 2001	0131026.7	
3. Full name, address and postcode of the or of each applicant (underline all surnames)	Fusion Antibodies Limited		
	PO Box 374 Belfast BT1 2WD		
Patents ADP number (If you know it)	08295982001		
If the applicant is a corporate body, give the country/state of its incorporation	United Kingdom		
4. Title of the invention	"Soluble Recombinant Protein Production"		
5. Name of your agent (If you have one)	Murgitroyd & Company		
"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)	Scotland House 165-169 Scotland Street Glasgow G5 8PL		
Patents ADP number (If you know it)	1198013	00001198015	
6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (If you know it) the or each application number	Country	Priority application number (If you know it)	Date of filing (day / month / year)
7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application	Date of filing (day / month / year)	
8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if: a) any applicant named in part 3 is not an inventor, or b) there is an inventor who is not named as an applicant, or c) any named applicant is a corporate body. See note (d))	Yes		

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Description

16

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Priority documents

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Statement of inventorship and right to grant of a patent (Patents Form 7/77)

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Any other documents (please specify)

11. I/We request the grant of a patent on the basis of this application.

Signature *Murgitroyd & Company* Date 28/12/2001  
Murgitroyd & Company

12. Name and daytime telephone number of person to contact in the United Kingdom

Róisín McNally

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0025114/28-Dec-01-04-10

# 1 "Soluble Recombinant Protein Production"

2

3 This application describes a methodology for the  
4 rapid production of soluble recombinant protein  
5 using high-throughput techniques. This method  
6 allows the cloning, expression and identification of  
7 soluble protein from a given target gene product by  
8 a rapid robust method. This ability to produce and  
9 analyse soluble recombinant protein in a rapid time  
10 period represents a significant advance in an area  
11 which has long been considered a significant  
12 production bottleneck in the field.

13

## 14 Introduction

15

16 The recombinant production of protein in bacteria,  
17 yeast, insect and mammalian cell lines has become a  
18 cornerstone of biological research and the  
19 biotechnology industry. Classical biochemical and  
20 chromatographical purification techniques usually  
21 produce inadequate amounts of a target protein to  
22 study its roles or actions. Even if enough of the

1 protein can be purified, it usually involves  
2 cumbersome amounts of starting material or tissue  
3 and many processing steps are taken before  
4 reasonable purification can be achieved.  
5

---

6 Recombinant expression of the target protein  
7 bypasses a lot of these problems. By introducing  
~~8 the target protein's gene template to a cell line or~~  
9 bacterial culture, induced overexpression can result  
10 in significant levels of that protein being  
11 produced. Large amounts of protein make the  
12 purification a lot simpler, but the addition or  
13 fusion of purification domains or tags allows for a  
14 relatively simple one-step purification using  
15 affinity chromatography resins.  
16

17 Bacteria, and more specifically, *E.coli* are ideal  
18 expression vehicles for the production of  
19 recombinant protein, as large amounts of foreign  
20 protein can be expressed in small culture volumes at  
21 low cost in comparison with other methods. However,  
22 the use of bacteria as expression hosts are not  
23 without problems. One of the most troublesome  
24 shortcomings of the use of *E.coli* is the production  
25 of the recombinant protein in an insoluble form,  
26 especially a problem when the target gene is non-  
27 bacterial. Generally, insolubility is the result of  
28 the production of protein that is not recognised by  
29 the folding enzymes, or chaperones, present in the  
30 bacterial cytoplasm. The unfolded or misfolded  
31 protein will attempt to decrease its own entropy to  
32 a minimum, and it is thought that in an effort to

1 hide or mask its hydrophobic residues from the  
2 aqueous environment, the protein molecules actually  
3 clump together. These clumps are insoluble and are  
4 called inclusion bodies. While in the form of  
5 inclusion bodies, the protein will have no  
6 biological activity and will be impossible to purify  
7 using affinity fusion tags. These inclusion bodies  
8 can be re-solubilised in chaotropic buffers such as  
9 8M urea or 6M guanidine hydrochloride, but then must  
10 be dialysed against physiological buffers in an  
11 effort to refold and regain biological function.  
12 Due to the individual characteristics of each  
13 protein, this is a slow and painstaking process that  
14 may never produce active or useful protein.  
15 Therefore, the ability to quickly produce and screen  
16 soluble protein in *E.coli* represents a major step  
17 forward in protein biochemistry.

18  
19 The following methodology presented describes a  
20 high-throughput process for the cloning, expression  
21 and analysis of recombinant soluble protein and  
22 protein domains. This process incorporates  
23 evaluation and comparison of many factors and  
24 conditions known to influence protein solubility at  
25 each step in order to guarantee generation of  
26 soluble recombinant protein.

27  
28 According to the present invention there is provided  
29 a method of producing a soluble bioactive domain of  
30 a protein the method comprising the step of  
31 selecting suitable soluble subunits of a protein and  
32 assessing the produced protein for desired activity.

1 Typically the method comprises the steps of analysis  
2 of DNA coding for the protein of interest to  
3 identify antigenic soluble domains, designing  
4 oligonucleotide primers to amplify DNA encoding the  
5 domain, amplifying DNA, cloning the DNA and

6 screening clones for correction orientation of DNA,

7 expressing DNA in expression strains, analysing

8 ~~expression products for solubility, analysing~~

9 products and production of soluble bioactive protein  
10 domain.

11

12 The invention is exemplified with reference to the  
13 following non limiting description and the  
14 accompanying figures.

15

#### 16 **Methods**

17

#### 18 **Template analysis and primer design**

19

20 The high throughput process begins with the analysis  
21 of the DNA coding for the protein of interest. Using  
22 software packages including Vector NTI (Informax,  
23 USA) and (<http://www.ncbi.nlm.nih.gov/BLAST/>) to  
24 identify complete domains within the protein that  
25 significantly increase the likelihood of  
26 antigenicity and solubility when expressed as a  
27 subunit of the original protein coding sequence. If  
28 possible three sub-domains are identified for  
29 processing. This has proven optimal to produce  
30 soluble protein with the majority of proteins  
31 expressed using this process.

32



1 The next step in the process is to design  
2 oligonucleotide primers to amplify these sub-  
3 domains. Primer design is aided by use of the design  
4 facility in Vector NTI the internet software package  
5 Primer3 (<http://www-genome.wi.mit.edu/genome>  
6 [software/other/primer3.html](http://www-genome.wi.mit.edu/genome_software/other/primer3.html), (Whitehead Institute  
7 for Biomedical Research). These packages allow full  
8 control over all aspects of primer design, ranging  
9 from primer length, homology to optimal annealing  
10 temperature of the PCR reaction itself. Typically  
11 the primers designed are 20 base pairs in length  
12 with annealing temperatures of between 58-62°C and  
13 are supplied by MWG-Biotech AG (Germany).

14

#### 15 PCR of Insert

16

17 The desired inserts are amplified using the primers  
18 designed specifically for that target gene. In  
19 order to maximise the chance of the PCR working  
20 without having to individually optimise each new  
21 primer set several standard conditions are used.  
22 Expand High Fidelity PCR system (Roche, Basal,  
23 Switzerland), is used for the reaction, working  
24 stocks of each primer are made (10pMol/μl) and each  
25 PCR is set up to be carried out under 3 different  
26 thermocycler conditions in every case.

27

- 28 1. A standard PCR programme using the recommended  
29 annealing temperature provided with the  
30 primers.
- 31 2. A standard PCR programme using 50°C as the  
32 temperature for annealing.



1 3. A touchdown PCR programme, where the annealing  
2 temperature starts at 65°C for 10 cycles and  
3 then gradually decreasing the annealing  
4 temperature to 50°C over the following 15  
5 cycles.

---

6  
7 The PCR products are then visualised on a 1.5%  
8 agarose gel stained with Ethidium Bromide (example  
9 in Figure 1) and the bands are cut out of the gel  
10 and purified using Mini elute gel extraction Kit  
11 (Qiagen, Crawley, England).

---

#### 12 13 Colony Screening for Inserts in Correct Orientation

14  
15 Ten of the transformants are screened using  
16 directional PCR using a primer that encodes for a  
17 sequence on the vector such as S Tag or GATA  
18 sequence, and then the complementary primer from the  
19 insert. A PCR mix is used - RedTaq DNA Polymerase  
20 (Sigma Aldrich, Dorset, England) and the  
21 thermocycler conditions used are the standard PCR  
22 programme using 50°C as the annealing temperature.

23  
24 The PCR products are visualised as shown in figure  
25 1. Positive clones are then inoculated in a small-  
26 scale culture (750µl) and grown at 37°C for 5 hours  
27 with shaking and then the plasmid DNA is extracted  
28 using SpinPrep 20 Plasmid Kit (Novagen, Madison,  
29 USA). The sequence of these clones is checked using  
30 sequencing.

31

1 We do not wish on most occasions to express the  
2 whole region of a target protein as this is only  
3 likely to cause insolubility problems, and it also  
4 presents less problems for our GMAG submissions.  
5 Therefore using bioinformatics software we intend to  
6 identify globular regions or domains of the target  
7 protein (if not already known) and amplify these  
8 regions.

9

10 Selection of clones and generation of constructs is  
11 shown in Figures 2 and 3.

12

### 13 Sequencing

14

15 The sequencing reactions are performed on the cloned  
16 inserts, using the Big Dye Terminator cycle  
17 sequencing kits (Applied Biosystems, Warrington, UK)  
18 and the specific sequencing primer run on a Peltier  
19 Thermal cycler model PTC225 (MJ Research Cambridge,  
20 Mass). The reactions are run on Applied Biosystems -  
21 Hitachi 3310 Sequencer according to the  
22 manufacturer's instructions. These sequences are  
23 checked to ensure that no PCR generated errors have  
24 occurred.

25

### 26 High Throughput Screening of Expression Clones

27

#### 28 1. Growth and Expression of Clones

29

30 For each construct four plasmids are selected and  
31 subcloned into *E. coli* strains (Rosetta(DE3)pLacI,  
32 Tuner(DE3)pLacI, Origami B(DE3)LacI). Following re-

1 transformation into the 3 *E. coli* expression  
2 strains, 4 colonies from each are chosen for high  
3 throughput screening. The ability to quickly screen  
4 clones for the production of the recombinant protein  
5 is absolute key to the successfulness of the present

6 Application high-throughput system.

---

7

8 Basic Setup of Protocol

---

9

10 1. Identify four positive clones on the NovaBlue  
11 transformation plates and miniprep and sequence.

12

13 2. Use these 4 minipreps to transform the three  
14 chosen expression strains. These are

15 a. Rosetta(DE3)pLacI

16 b. Tuner(DE3)pLacI

17 c. Origami B(DE3)LacI

18

19 3. This will generate 12 agar selection plates for  
20 each insert being studied. Take 4 colonies per  
21 plate and use it to inoculate in duplicate two wells  
22 in the 96 well plate and grow this as the primary  
23 culture. The setup of the plates is as follows is  
24 describe in the flow diagram (Figure 8).

25

26 The colonies are used to inoculate duplicate wells  
27 in a 96 well plate. Each well contained 200 µl of  
28 LB broth with the appropriate antibiotics. Each  
29 plate is dedicated to one strain of *E. coli* which  
30 alleviates the problems of different growth rates.  
31 The necessary controls are also included on each  
32 plate. The plates are then grown up at 37°C, with

1 shaking, until log phase is reached. This is the  
2 primary plate. From the primary plate a secondary  
3 plate is seeded using 'hedgehog' replicators. This,  
4 secondary, plate is then grown up to log phase,  
5 chilled to 16°C for 1 hour then induced with IPTG,  
6 to a final concentration of 1 mM. The plates are  
7 then grown up at 16°C overnight. The primary plate  
8 is stored at 4°C until the process is complete.

9

## 10 2. Harvesting and Lysis Of Cells

11

12 The contents of each well in the 96 well plate are  
13 transferred into a Millipore 0.65 µm multi-screen  
14 plate. The plate is placed on a manifold and a  
15 vacuum is applied. This draws off the culture  
16 medium to waste. The cells are then be washed with  
17 PBS (optional), again the vacuum is applied to  
18 remove the PBS. The multi-screen plate is removed  
19 from the manifold and bacterial cell lysis buffer  
20 (containing DNase) (50 µl) is added to each well.  
21 The plate is incubated at room temperature for 30  
22 minutes with shaking to facilitate lysis of the  
23 cells. A fresh 96 well microtitre plate (ELISA  
24 grade) is placed inside the vacuum manifold and the  
25 multi-screen plate is placed above it. When a  
26 vacuum is applied the contents of each well are  
27 drawn into the micro-titre plate below. The vacuum  
28 only needs to be applied for 20 seconds. The  
29 collected lysate contains the soluble fraction of  
30 expressed protein. To analyse the insoluble  
31 fraction, urea buffer (50 µl) is added to the wells  
32 of the multi-screen plate and it is incubated at

1 room temperature for 30 minutes with shaking. The  
2 contents of each well are removed manually using a  
3 multi-channel pipette and are either stored or used  
4 directly in a dot blot (see next stage).

5

---

6 ~~3. Analysis of the Soluble and Insoluble Fractions~~

---

7

---

8 ~~The soluble fraction of the cell lysate are analysed~~

---

9 either by direct ELISA or by dot blot:

10

11 ELISA : 2X sodium carbonate buffer (50 µl) is added  
12 to each well and the plate is incubated at 37°C for  
13 1 hour. The plate is inverted to remove the  
14 contents and subsequently blocked with 3% BSA in  
15 TBS-T (300 µl/well). The plate is incubated at 37°C  
16 for 1 hour. The blocking reagent is removed as  
17 before and the wells are probed with anti-polyHis-AP  
18 antibody for 30 minutes at 37°C. The plate is  
19 washed three times over 10 minutes with TBS-T and  
20 substrate is added to each well to disclose soluble  
21 expressed protein.

22

23 Dot Blot: Dot blot analysis can be used for both  
24 soluble and insoluble fractions. The samples are  
25 applied to the dot blot apparatus housing  
26 nitrocellulose membrane. The insoluble fraction dot  
27 blots are washed with TBS (200 µl) to remove excess  
28 urea. The membranes are blocked for 30 minutes at  
29 room temperature (3% BSA in TBS) then probed with  
30 anti-polyHis-AP antibody for 30 minutes at room  
31 temperature. The blots are then washed once with  
32 TBS, 5 minutes at room temperature, then once with

1 alkaline phosphatase buffer, 5 minutes at room  
2 temperature. Positive clones are then identified  
3 following the addition of alkaline phosphates  
4 substrate.

5

#### 6 **Analysis of Products**

7

8 Four proteins (arbitrarily named A, B and C) are  
9 analysed using the above method. Colonies  
10 harbouring plasmids expressing each of the 4  
11 proteins are grown up in a 96 well plate and  
12 harvested as described. Soluble and insoluble  
13 fractions are prepared and analysed both by dot and  
14 Western blot.

15

#### 16 **Isolation of Insoluble Expressed Protein**

17

18 Analysis of the insoluble material provides a  
19 complete picture of the expression of recombinant  
20 protein. To facilitate analysis of the insoluble  
21 fraction, urea buffer (50 µl) was added to each of  
22 the wells of the multi-screen plate. The plate was  
23 incubated at room temperature for 30 minutes with  
24 shaking. The contents of each well were then  
25 removed manually using a multi-channel pipette and  
26 were either stored or used directly in a dot blot  
27 (as described).

28

29 Of the 4 proteins investigated one, namely protein  
30 A, is found to be expressed in a soluble form as  
31 confirmed by dot blot of the soluble cell lysate  
32 fraction. Soluble product is not observed with the

1 other proteins investigated as confirmed by dot blot  
2 analysis of both the soluble and insoluble fractions  
3 (refer to figures 4 and 5).  
4

5 These results are further confirmed by Western blot  
6 analysis of the expression from three colonies for  
7 each of the proteins (refer to figure 6).  
8

9 The primer design requires the upstream primer to  
10 contain an ATG start codon and the downstream primer  
11 to contain the antisense for a stop codon. We have  
12 added our own requirements to this primer design to  
13 meet with the high-throughput requirements of the  
14 present Application system. These requirements are,  
15

16 A. In the upstream primer, the start ATG codon  
17 should be preceded with a G or a A. This simple  
18 addition means that the same forward primer can be  
19 used for the insertion of an insert into all vectors  
20 (the G fulfils a AAG Lys codon (or AAA if another A  
21 used) at the end of the enterkinase site in the GST  
22 tag).  
23

24 B. The stop codon to be used is TAA, therefore the  
25 reverse primer should start with the sequence ATTA,  
26 with the additional T aiding the efficiency of the  
27 stop codon.  
28

29 C. Incorporation of histags is achieved by the  
30 addition of the coding region in the primer, forward  
31 or reverse as required.  
32



1 D. The addition of a TAG stop codon is essential  
2 in the native reverse primer. This is to act as a  
3 stop codon when cloned in the opposite direction as  
4 to what is required when using the vector. When  
5 cloning into the vector, an overexpressed protein  
6 will be produced matter what orientation the insert  
7 is in (due to the expression of the GST tag). On  
8 gels and blots it would be very hard to tell a  
9 positive clone from a negative if the target insert  
10 is very small (due to the translation of rubbish  
11 from the insert in the wrong orientation). The  
12 addition of this TAG in the reverse primer means  
13 that when the insert is in the wrong orientation,  
14 then no translation beyond the GST tag will occur.  
15 This should make positive identification of positive  
16 expression much easier on gels and blots.

17  
18 1. Gradient PCR. The use of the 96 well block  
19 allows the use of an optional annealing gradient.  
20 Therefore, we can tailor the block to suit the  
21 calculated annealing temperature for each reaction  
22

23 2. Touchdown PCR. This starts at a annealing  
24 temperature likely to be above the true value and  
25 decreases slowly with cycles, so that can increase  
26 the chance of amplifying the product if it is likely  
27 to be problematic.

28  
29 3. 50°C annealing temperature. This is a program  
30 of last resort. It is likely to produce more than  
31 one band, but can be invaluable with problem inserts  
32

1 Following PCR, the products are then analysed by  
2 agarose electrophoresis and also purified by gel  
3 excision as this minimizes the chance of carrying  
4 template plasmid clones through into the cloning  
5 procedures, which may result in false positives on  
6 the transformation selection plates.

7

8 ~~Positive clones can then be propagated to perform a~~  
9 plasmid miniprep, which subsequently can be used for  
10 DNA sequencing and transformation into proper  
11 expression strains. It is possible to induce  
12 expression in NovaBlue cells, but this is a slow  
13 growing strain that it not protease deficient unlike  
14 some of the tailormade B strains that are available  
15 on the market.

16

#### 17 High Throughput Expression Evaluation

18

19 The ability to quickly screen clones for the  
20 production of the recombinant protein is absolute  
21 key to the successfulness of the whole present  
22 Application system. We select 4 clones from each  
23 cloning transformation, and after miniprep'ing, to  
24 analyse 4 colonies in duplicate per plate.

25

26 1. This format is performed as each plate is  
27 dedicated to one strain, as to mix strains on the  
28 one plate is awkward due to the different growth  
29 rates. Therefore, each plate can be made up for the  
30 analysis of 2 constructs. This initial plate is  
31 called the primary plate and is grown at 37 °C until  
32 the cultures reach late log phase

1

2 2. From this plate, secondary plates are seeded  
3 using 'hedgehog' replicators, and these are again  
4 grown up to log phase, then induced with IPTG and  
5 left to grow overnight at 25°C

6

#### 7 Dot-Blot Analysis of Expression

8

9 Once we have induced and grown the cultures, the  
10 presence of recombinant protein, and its solubility  
11 is determined. This is performed using dot-blot,  
12 then screening the dot blots with anti-histag  
13 antibody

14

15 1. Firstly, the grown plate is spun at 4K g to  
16 pellet the bacterial cells and the supernatant is  
17 simply removed by inversion of the plate

18

19 2. The cell pellet is resuspended in bacterial  
20 lysis buffer and after lysis, spun again to separate  
21 soluble and insoluble protein

22

23 3. A portion of the supernatant (soluble fraction)  
24 is taken and applied to a dot blot for total  
25 analysis of soluble protein

26

27 4. Resuspended pellet is then applied to a second  
28 dot blot for the analysis of insoluble protein for  
29 each of the clones

30

31 This analysis provides a picture of the expression  
32 status of the clones on each plate. Using these we  
33 then select positive soluble protein expressing

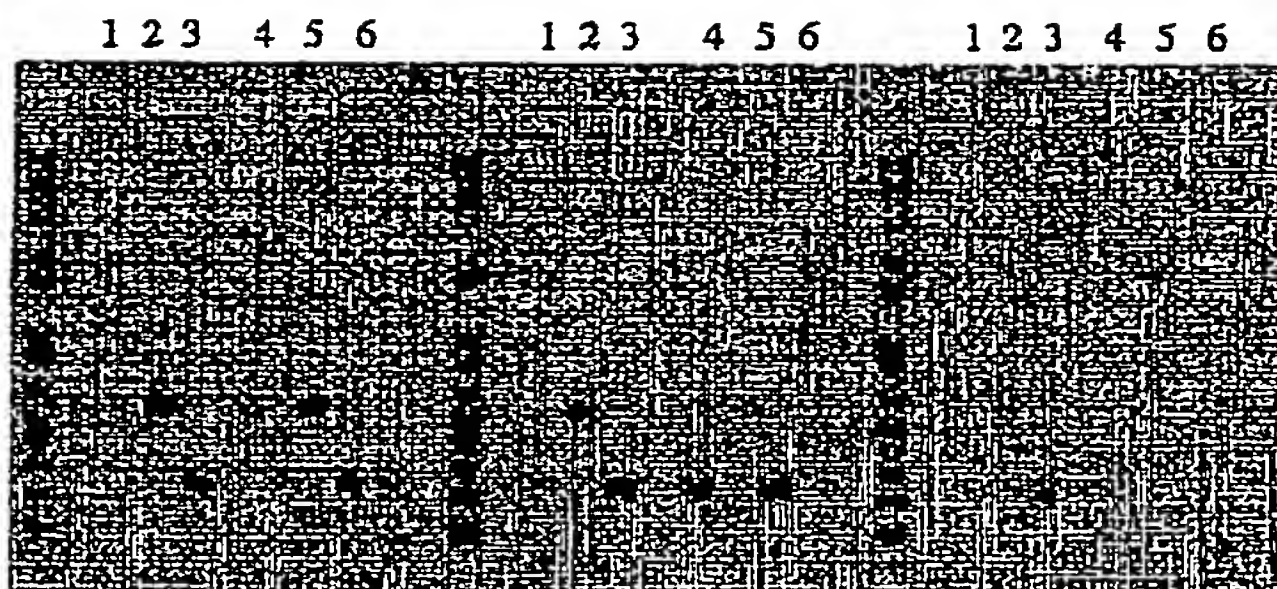
1 clones and scale their growth up to 5 ml scale,  
2 using the saved primary plate as an inoculum.  
3  
4 These will be induced and grown in a similar manner  
5 for the preparation of a small scale NiNTA prep to  
6 identify whether or not the soluble protein will  
7 bind to NiNTA, thus facilitating purification. This  
8 ~~can be checked by SDS-PAGE and western blotting of~~  
9 the samples pre and post purification with the NiNTA  
10 columns. At this point have identified good  
11 candidates for large scale expression and  
12 purification.  
13  
14 Figure 7 shows a putative timetable for the process  
15 from the time that the order is placed to expression  
16 of immunisation-ready protein.  
17

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Figure 1

PCR insert gel.  
Labelled 123 456

1=Fragment 1 minus DMSO  
2=Fragment 2 minus DMSO  
3=Fragment 3 minus DMSO  
4=Fragment 1 plus DMSO  
5=Fragment 2 plus DMSO  
6=Fragment 3 plus DMSO



(6 bands - each set are separate by molecular weight markers), Set 1 are for the 50°C reaction, set 2 are the actual annealing temp for primers, and set 3 are the touchdown PCR.

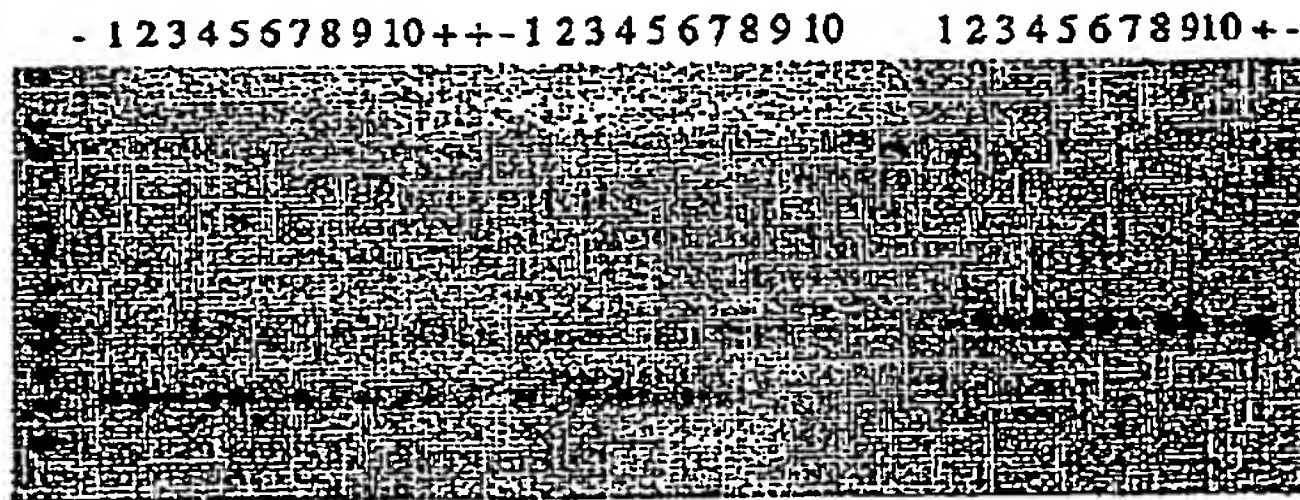
2/8

**Figure 2****Colony PCR gel****Labeled 1-10.** these labels correspond to the colonies screened ie: 10 from each plate. +=Positive control**-**negative control.

first 1-10 are colonies from fragment 1

The second set labelled 1-10 are colonies from fragment 3

The third set labelled 1-10 are colonies from fragment 2.



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Figure 3

These constructs are generated:

1.



N-terminal Histag

target protein

2.



target protein

C-terminal Histag

3.



GST tag

Histag

target protein

Primer design using guidelines in the previous section can be facilitated quickly and easily using Vector NTI and Primer3 software.

In the situation described below, where a protein contains 2 globular domains, we could amplify some 9 constructs, as demonstrated below. However, we chose the 4 most appropriate.

(globular regions are shown in light grey)



The arrows demonstrate the points at which we wish the primers to anneal to the template cDNA.  
(draw 4 possible products)

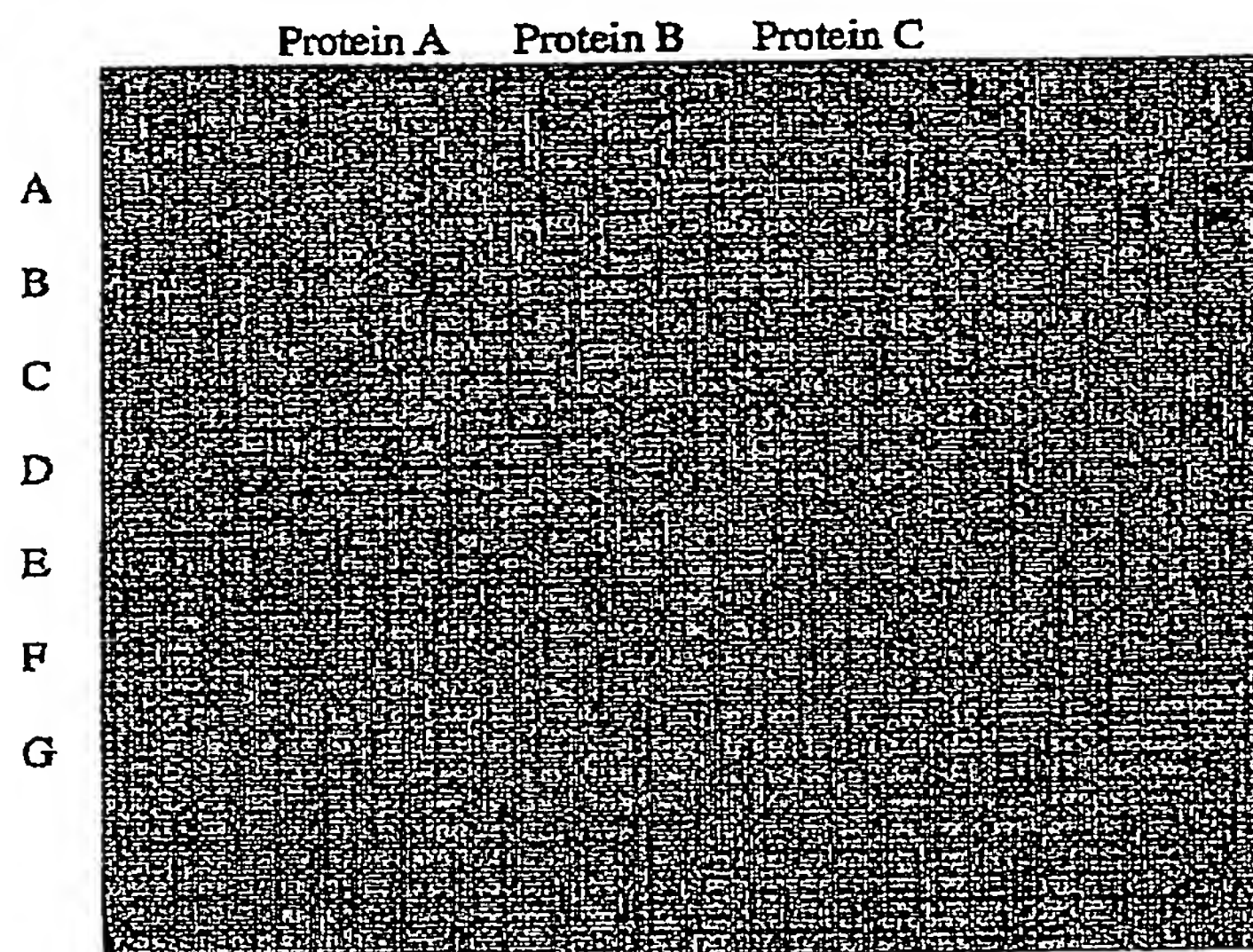
Using our requirements for the design of the primers, we can effectively produce 4 different inserts using only 4 primers, as opposed to the many more it would require based on the strategies for each of the vectors on their own.

The inserts shown on the diagram can be cloned into any vector with the use of a TA cloning kit, the use of a high fidelity *Taq* is required, so that A overhangs are left on the PCR products. The other three native fragments are specially designed to be inserted into the vector, which requires the use of blunt ended products, which can be produced when using a proof-reading polymerase such as *Pwo* or *Pfu*.



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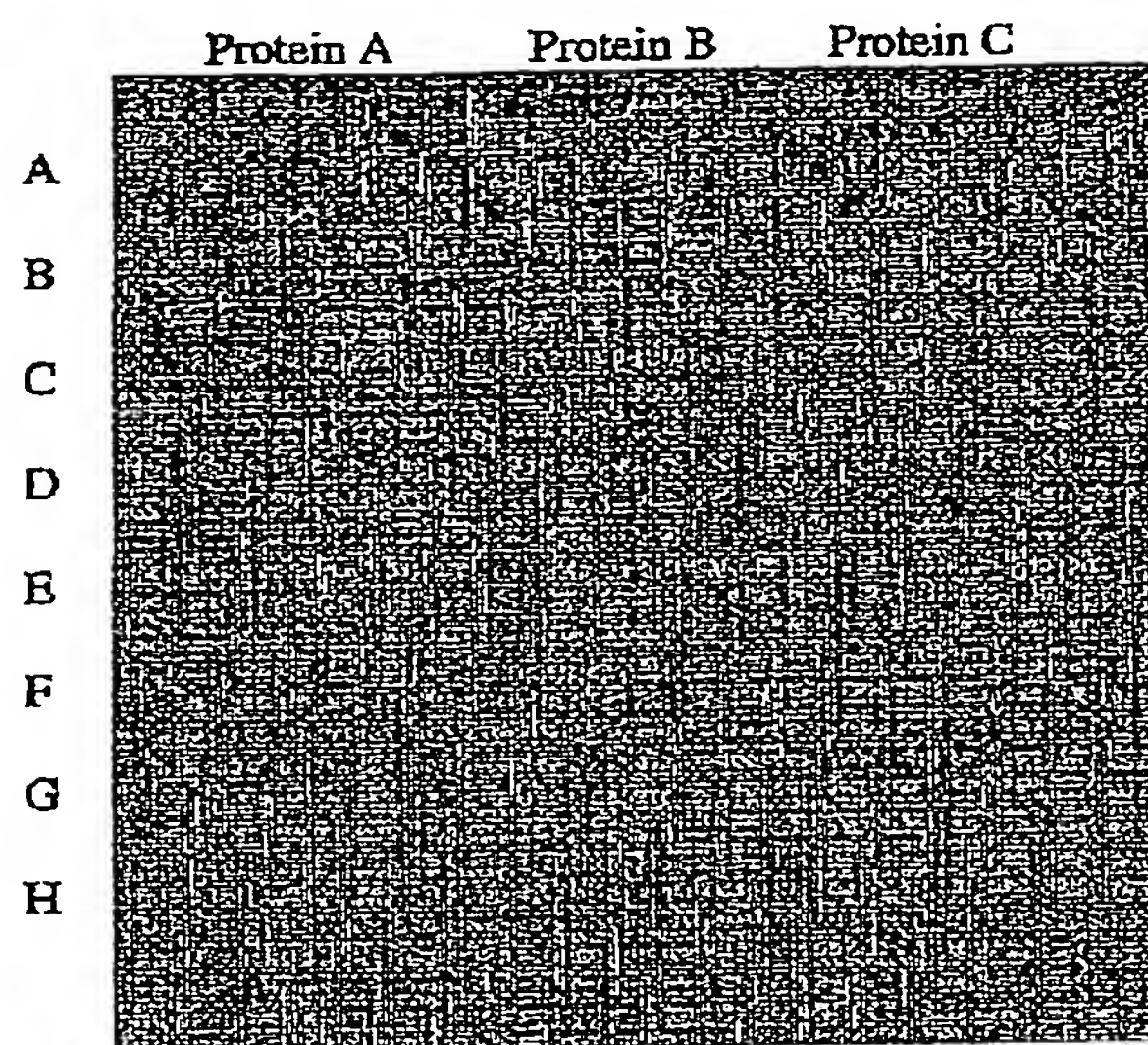
Figure 4 (Dot blot analysis of soluble fractions)



Rows A-F represent duplicates of 6 clones  
Row G -lysis buffer only

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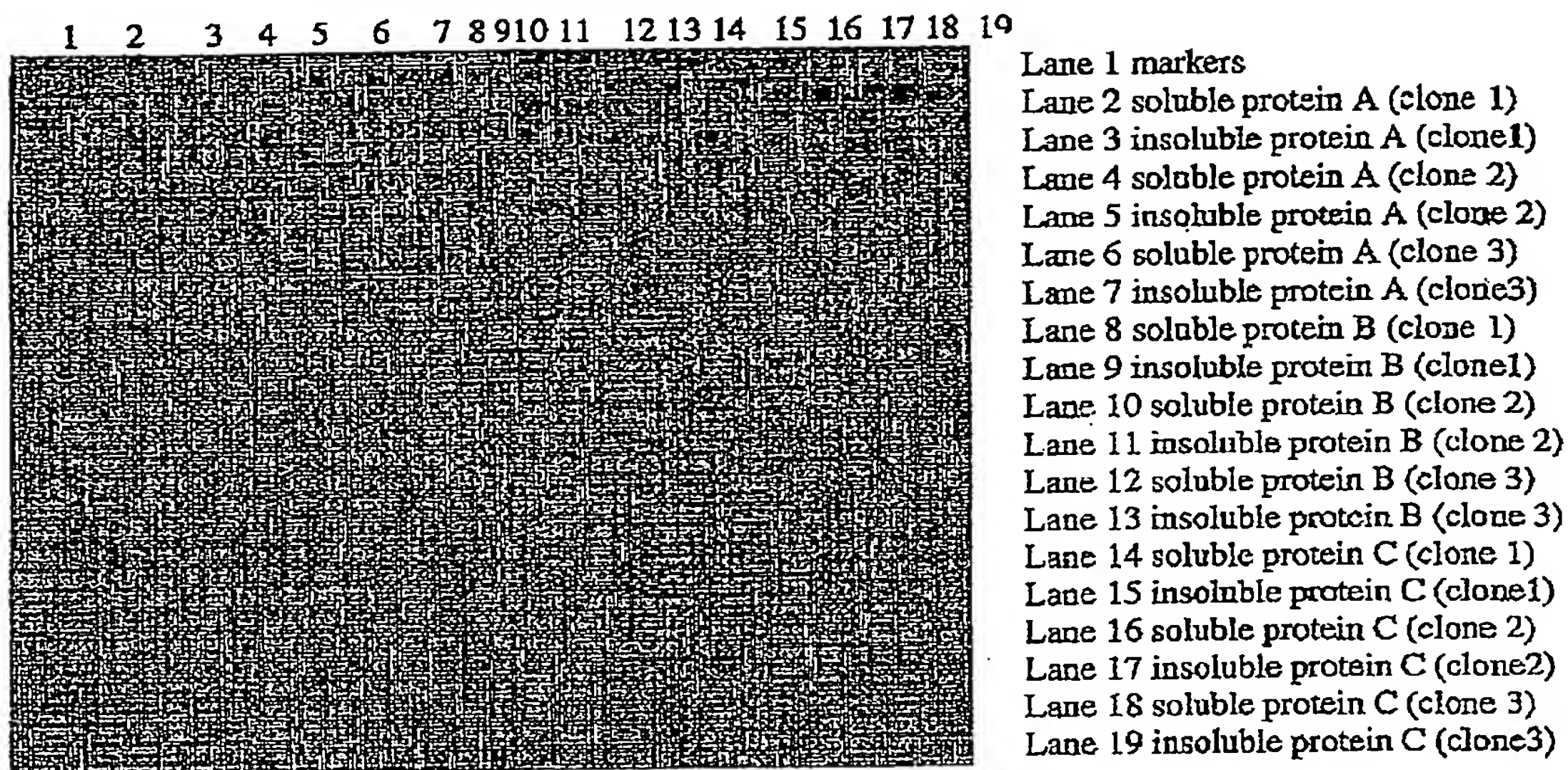
Figure 5 (Dot blot analysis of insoluble fractions)



Rows A-F represent duplicates of 6 clones  
Row G lysis buffer only  
Row H urea buffer

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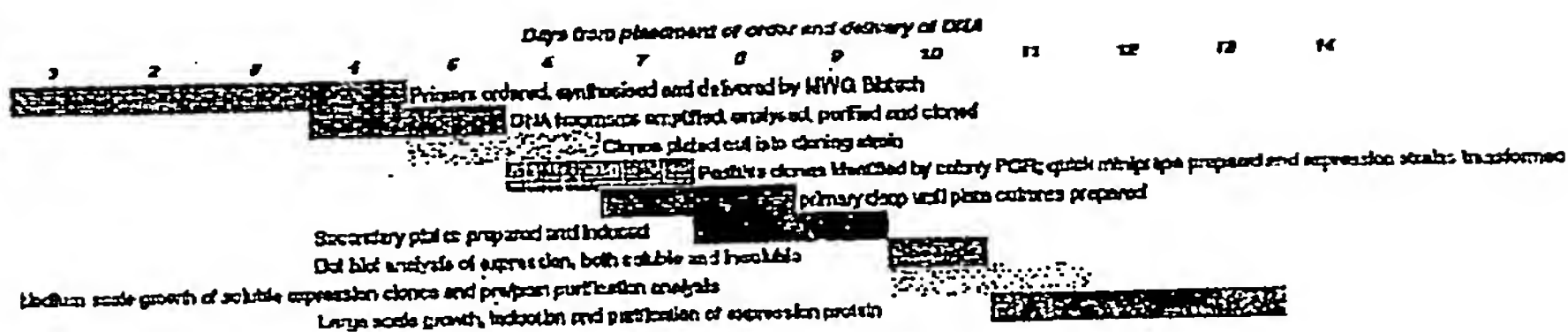
Figure 6 (Western blot analysis)



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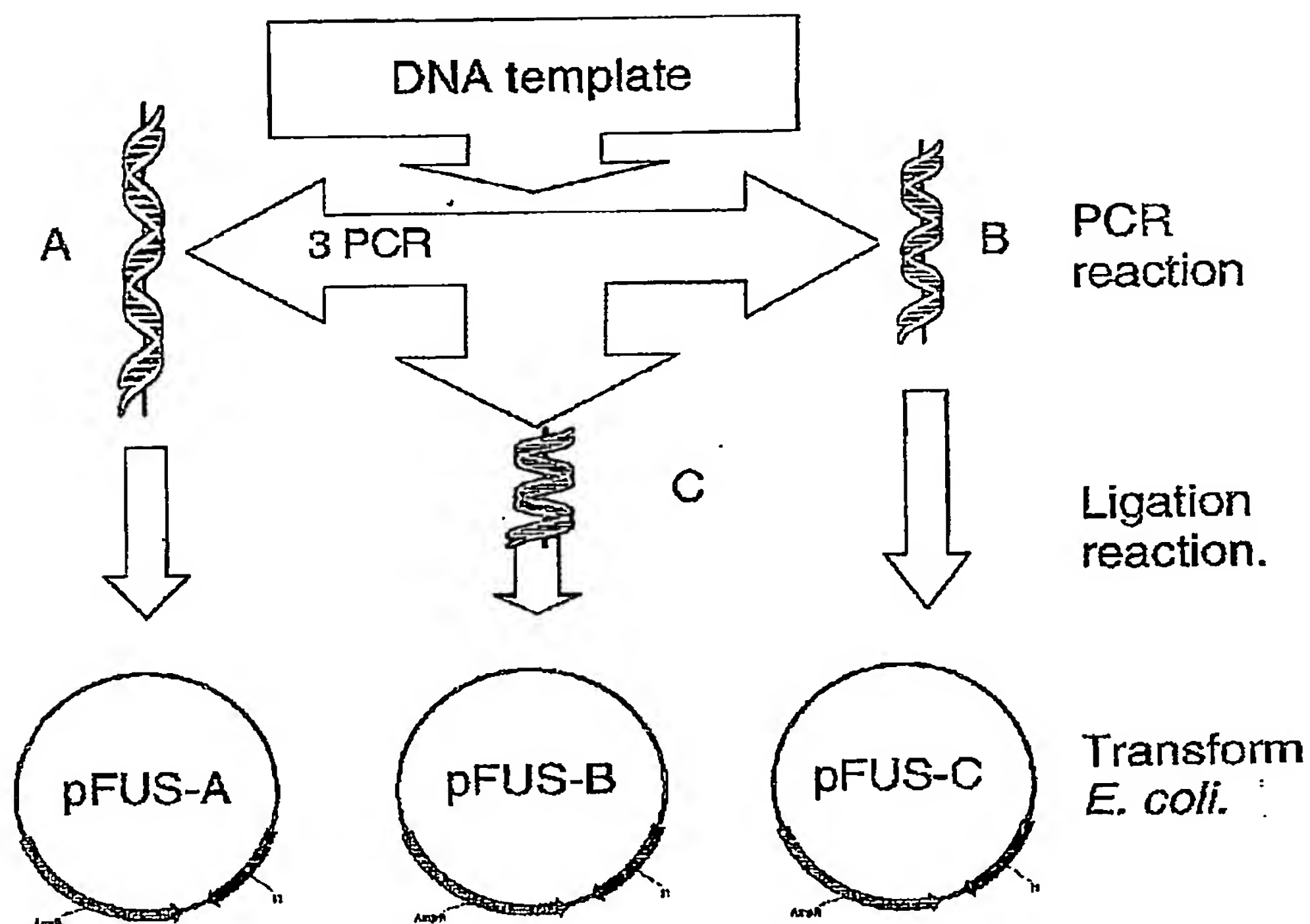
Figure 7

## Timetable for Production of Protein

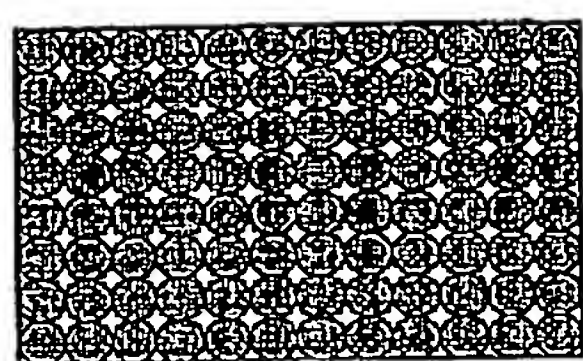


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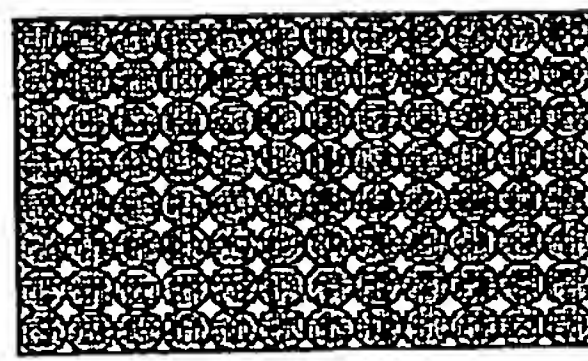
Figure 8



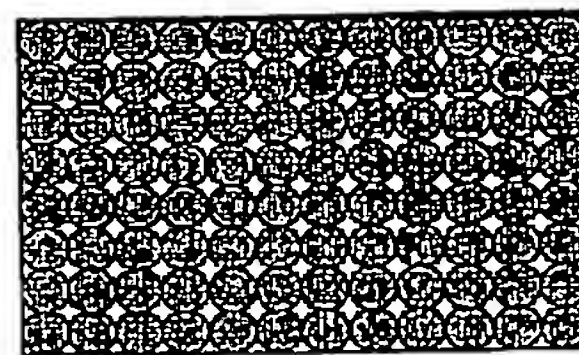
4 clones from the first transformation experiment are selected and used to transform 3 *E. coli* expression strains. 4 colonies from each expression strain are screened in duplicate for soluble proteins, resulting in 3 x 96 clones screened from each template.



Tuner(DE3)pLacI  
I



Origami(DE3)pLacI  
expression cells



Rosetta(DE3)pLacI  
expression cells

Flow chart of the fusion antibodies  
high-throughput process

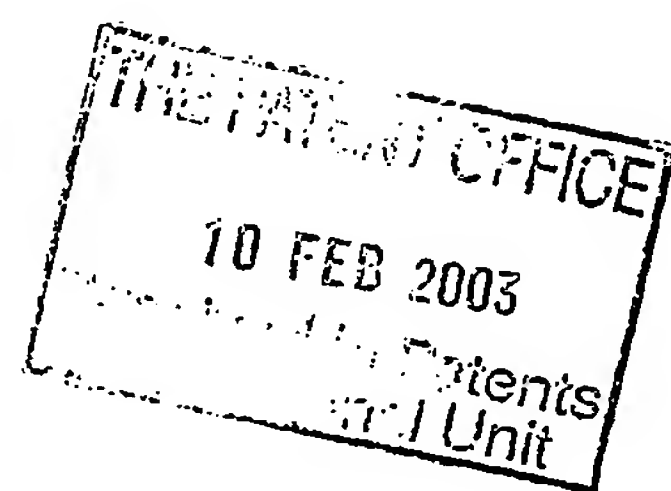


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P. T.

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